REMARKS

Independent Claims 1 and 19 have been amended to address the 35 U.S.C. § 112 issue raised by Examiner in the Official Action. In particular, Claim 1 has been amended to specify that what is being claimed is a method to aid in detecting a predisposition in an individual of developing preeclampsia, and thereby indicate individuals at risk of developing preeclampsia, wherein a lower than normal level of a peptide having the amino acid sequence set forth in SEQ ID NO:1 correlates with magnesium binding defect. Support for this amendment can be found in the instant specification, for example at [0054] Independent Claim 19 has also been amended to specify a method for monitoring progress in treatment of preeclampsia comprising comparing levels of peptide before and after treatment, wherein a lower than normal level of peptide correlates with presence of the magnesium binding defect. New independent claims 31 and 36 (and Claims 32-35 and 37-40 dependent thereon) have been presented, which like claim 1, are also directed to a method to aid in detecting a predisposition to developing preeclampsia by measuring levels of peptides having amino acid sequence set forth in SEQ ID NOs: 2 and 4, respectively. It is believed that none of these amendments constitute new matter and their entry is requested.

Rejection under 35 U.S.C. § 112

The Examiner has rejected claims 1, 3-6 and 19 under 35 U.S.C. 112, first paragraph, and asserted that the specification is enabled only for detecting lower level of peptide in plasma associated with essential hypertension, but not for preeclampsia. In particular, it was asserted that the specification is deficient in teaching how to use the invention because Applicant has not demonstrated that measurement of the levels of the peptides of SEQ ID NOs: 1, 2, and 4 is indicative of the magnesium binding defect. (Official Action page 4) Applicant respectfully

disagrees and traverses this rejection. As discussed below in detail, the specification provides evidence demonstrating that levels of peptides of SEQ ID NOs: 1, 2, and 4 actually correlate with the presence of the magnesium binding defect.

The Specification Provides Evidence that a Lower that Normal Level of Peptides of SEQ ID NOs: 1, 2 and 4 Correlates with the Magnesium Binding Defect

Evidence that lower than normal levels of the peptides of SEQ ID NOs: 1, 2, and 4 (the "Peptides") are correlated with the magnesium binding defect is provided throughout the instant specification, for example at paragraphs [0018], [0034], [0042-0047], [0056] and Examples 2, 3, and 7. Specifically, the specification reports the findings of Wells and Agrawal (1991) that the concentrations of tightly-bound magnesium in salt-sensitive, hypertensive rat models SHR and SS/Jr were lower than in the normotensive rat model (WKY) and that the magnesium binding defect occurred in both the SHR and SS/Jr rats, but did not occur in the WKY rats. (paragraph [0042]) This reference further teaches that the binding of magnesium in erythrocytes of SHR rats that have the magnesium binding defect, was increased to the normal value when erythrocytes displaying the magnesium binding defect were incubated with normal plasma. In addition, the specification reports (paragraph [0042]) the findings of Mattingly, Brezezinski and Wells (1991) that binding of magnesium by erythrocyte membranes was returned to normal by incubating erythrocytes from essential hypertensive patients with blood plasma from normotensive subjects. Thus these references teach that a component of normal blood plasma promotes magnesium binding to plasma membranes, and that the concentration of this promoter/component is at a lower level (or not present in) the plasma of rats with the magnesium binding defect.

The instant specification reports Applicant's discovery that the Peptides are components in normal plasma that promote magnesium binding to plasma membranes and thus prevent the magnesium binding defect, paragraph [0047]. The discoveries reported provide evidence of the correlation between the levels of Peptides and the magnesium binding defect, and make possible a method to aid in detecting a predisposition to developing preeclampsia, paragraph [0054]. Specifically, objective evidence of this correlation is reported in the specification through demonstration that the levels of the Peptides in plasma from rat models in which the magnesium binding defect was observed are lower than normal levels of Peptides found in rat models without the magnesium binding defect. Further evidence substantiating this correlation through examples demonstrating the *in vivo* effectiveness of the intravenous administration of the Peptides in increasing magnesium binding (and in reducing blood pressure) (Example 2; paragraph [0047]). Additional evidence of the correlation is provided in the specification: 1. demonstration that binding of magnesium to erythrocyte membranes from essential hypertensive patients was returned to normal by *in vitro* incubation with the Peptides (Example 3), and 2. demonstration that the Peptides promote magnesium binding in magnesium deficient erythrocytes (Example 7).

To summarize, the instant specification provides evidence that a significantly lower than normal level of Peptides correlates with the presence of magnesium binding defect, and, together with the teachings of the specification that preeclampsia is associated with the magnesium binding defect, the specification enables one skilled in the relevant art to make and use the claimed invention.

The Official Action further states that the specification does not provide a working example for the measurement of Peptides from a body fluid. (Official Action page 4) Applicant asserts

that working examples are not required and that prophetic examples may be used. Atlas Powder Co. v. E.I. du Pont de Nemours & Co., 224 USPQ 409 (Fed. Cir. 1984) (The use of prophetic examples does not automatically make a disclosure non-enabling.) An enabling disclosure is all that is required and neither actual embodiments or working examples are necessarily required as long as the disclosure is enabling. In re Long, 151 USPQ 640 (C.C.P.A. 1966). It is urged that the specification describes the manner of carrying out the method of the invention, including detailed teachings which describe exemplary methods employing routine immunochemical procedures known in the art to detect Peptide levels in body fluid. Paragraphs [0057-0110]. Thus, the specification enables a skilled artisan to practice the claimed invention using no more than reasonable routine experimentation. *In re* Swartz, 56 USPQ2d 1703 (Fed. Cir. 2000) ("To satisfy the enablement requirement of § 112, a patent application must adequately disclose the claimed invention so as to enable a person skilled in the art to practice the invention at the time the application was filed without undue experimentation. Enzo Biochem, Inc. v. Calgene, Inc., 52 USPQ2d 1129, 1136 (Fed. Cir. 1999)."). See also, In re Wands, 8 USPQ2d 1400, 1402 (Fed. Cir. 1988). (Working examples are but one factor in determining the extent to which claims are enabled.); In re Stephens, 188 USPQ 659 (C.C.P.A. 1976) (All that is required is sufficient working procedure for one skilled in the art to practice the claimed invention without undue experimentation.)

The Official Action cites Page et al. as further basis for the rejection. Specifically, the Examiner asserts that Page et al. makes the finding of a decreased level of Peptide as indicative of preeclampsia unclear, and that Page et al. supports a conclusion that the correlation between level of Peptide and preeclampsia is unpredictable. Applicant strongly disagrees. Several points are made here in response to this basis for rejection. First, Page et al. reports that levels of

neurokinin B (NKB) in plasma of pregnant women with preeclampsia were elevated. The Official Action (page 5) asserts that the "intended patient population for [the method of the invention] is pregnant women who do not have preeclampsia." Applicant urges that this is an incorrect interpretation of the claim language. Applicant has amended Claim 1 in an attempt to clarify that the method of the invention is not limited in applicability to pregnant women.

Second, the Examiner admits that Page et al. is silent as to the levels of Peptides. (Official Action page 5) The claimed invention involves the measurement of levels of Peptides, not levels of NKB (or other tachykinins). There is no limitation in the claims requiring that the method measure the levels of tachykinins or tachykinin peptides. It is urged that the Official Action has referred to a limitation which is not present in the claims.

Third, a specification that discloses information on how to make and use the invention must be accepted unless the Patent Office provides sufficient reason to doubt the accuracy of the disclosure. Ex parte Bhide, 42 USPQ2d 1441, 1147 (Bd. Pat. App. & Int'f 1996) ("A specification which contains a statement of the manner and process of using the invention in terms which correspond in scope to those used in defining the subject sought to be patented must be taken as in compliance with the 'how to use' requirement of the first paragraph of 35 USC Section 112 unless there is reason to doubt the objective truth of the statement. In re Brana 34 USPQ 1436 (Fed. Cir. 1995); In re Marzocchi, 169 USPQ 367 (CCPA 1971).") For the reasons discussed herein, Page et al. simply does not provide sufficient reason to doubt the accuracy of the disclosure of the present specification.

Fourth, the Examiner admits that since Page et al. is silent as to the levels of Peptides, it is possible that detection of lower levels of the Peptides is correlated with preeclampsia, and invites Applicant to provide actual evidence demonstrating the claimed invention is contrary to that

which is taught by Page et al. (Official Action page 5) As set forth in detail above, the specification provides the required evidence demonstrating that Peptide levels actually correlate with the presence of the magnesium binding defect, and further that preeclampsia is associated with the magnesium binding defect. As such, contrary to the assertions in the Official Action at page 6, the specification demonstrates that detection of lower than normal levels of Peptide has predictive power concerning individuals at risk for developing preeclampsia.

In summary, in as much as Page et al. reports only findings of NKB levels in pregnant women with preeclampsia, does not disclose or suggest the levels of Peptides, and further, because the instant specification provides adequate disclosure for one in the art to make and use the claimed invention, Page et al. does not make the correlation between the levels of Peptides and preeclampsia "unpredictable".

The Official Action also cites the Merck Manual of Diagnosis and Therapy, 17th edition (1999) ("Merck") for reporting that the etiology of preeclampsia was unknown. Applicant does not understand this basis for rejection. It appears that Merck may have been cited as representative of the state of the art of the invention. However, that the Merck reference did not report detection of Peptides as predictive of preeclampsia, at the time the instant application was filed, in no way makes the discoveries reported in the specification, or the novel method of the present claims, unpredictable. Applicant urges that the state of the art of concern, that of peptide detection in body fluid, is sufficiently predictable that one of ordinary skill in the relevant art could use the specification as a guide to practice the invention.

Applicant notes the statement in the Official Action that "a predisposition...need[s] to indicate at a minimum that it is more likely than not that this individual will develop preeclampsia at a later date." Applicant does not understand form where this conclusion arose

and urges that this is an incorrect interpretation of the claim language. The definition for the word 'predisposition' provided in The American Dictionary of the English Language, 4th edition (2000) is "the state of being predisposed: tendency, inclination, or susceptibility"; and the alternative definitions provided in The American Heritage Stedman's Medical Dictionary (2002) are "1. The state of being predisposed, 2. A condition of special susceptibility, as to a disease." (Applicant herewith provides a print-out from the reference source of these definitions, website Dictionary.com.) It is urged that the definition proposed in the Official Action is not the ordinary and accustomed meaning for 'predisposed' recognized by one of skill in the art.

In summary, Applicant urges that the present specification provides an enabling disclosure that demonstrates both, the correlation of lower than normal levels of peptides of SEQ ID NOs: 1, 2, and 4 with the magnesium binding defect, and the association of preeclampsia with magnesium binding defect, and therefore, the absence of a working example for the measurement of Peptides in body fluid does not make the present specification non-enabling. Furthermore, it is urged that neither the report in Page et al. that levels of NKB are elevated in pregnant women, nor the report in Merck that, at the time of the discoveries reported in the instant specification, the etiology of preeclampsia was unknown, can support a finding that the correlation of lower than normal levels of Peptide with risk of developing preeclampsia is unpredictable. As such, Applicant asserts that the correlation between levels of Peptides and the risk of an individual developing preeclampsia is not unpredictable and that a skilled artisan could, in view of the teachings of the present specification, use and carry out the claimed method without undue experimentation.

In view of the teachings in the specification of the correlation of lower levels of peptides having amino acid sequences set forth in SEQ ID NOs: 1, 2, and 4, and the association of the

magnesium binding defect with the risk of developing preeclampsia, and in further view of the comments set forth above, it is urged that the claims are fully enabled and it is requested that the rejection under 35 U.S.C. § 112, first paragraph, be withdrawn.

New Grounds of Rejection Under 35 U.S.C. § 112

The Examiner has rejected Claims 1, 3-6 and 19 under 35 U.S.C. 112, first paragraph, on the basis that the specification is not fully enabling, commensurate with the scope of the claims.

The Examiner has acknowledged that the instant specification is enabling for an antibody that specifically binds the sequence of SEQ ID NO:1 and SEQ ID NO:4. However, Examiner asserts that based on the teachings of Couraud et al., the specification is not enabling for an antibody that specifically binds SEQ ID NO:2. Applicant submits that the specification would enable one of ordinary skill in the art to practice the full breadth of the claimed invention and respectfully traverses the Examiner's rejection.

Only routine experimentation is required to generate an antibody that binds to the peptide of SEQ ID NO:2.

The teachings of the Harlow et al. publication (1988) referred to in the Official Action (page 5) had been revised and replaced, as of the filing date of the present application, by Harlow et al. (1999) (Using Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Press). Harlow et al. (1999), therefore describes at least minimum knowledge in the art as of the filing date of the instant application. Applicant herewith provides referenced portions of this publication.

> The region of an antigen that interacts with an antibody is defined as an epitope. An epitope is not an intrinsic property of any particular structure, as it is defined only by reference to the binding site of an antibody. The size of an epitope is governed by the size of the combining site. From X-ray studies of the structures of cocrystals between small antigens bound to

> antibodies, the size of the combining site was though to be relatively small. . ..Later work using larger antigens shows that the area of these antigens in close apposition to the antibody can be quite large, occupying as much as 500-700 A^2 and often involving contacts with multiple CDRs, and many times establishing contact with all six. Although these studies have shown that epitopes can be much large than originally thought, it is still clear that high-affinity antibodies can be raised to small epitopes. (page 25)
>
> There are many potential methods for mapping and characterizing the location of epitopes on proteins, ranging from solving the crystal structure of the antibody-antigen complex to analysis of vast libraries of random peptide sequences. . .Three of the simplest, most widely applicable, and most robust assays are competition assays, gene expression assays, and synthetic peptide-based assays. (page 384)

Table 11.2 (page 384) of Harlow et al. teaches that synthetic peptide based assays can be used to map linear epitopes of 3-15 amino acids.

Harlow et al. (1999) further teaches use of monoclonal antibodies in immunoprecipitation technique (page 226-227) noting that an epitopes are "often comprised of only 4 or 5 amino acids" (page 227, second paragraph).

Applicant thus urges that only routine experimentation using art recognized methods would be required to generate the claimed antibodies. The fact that some amount of work must be performed to reach a successful end does not mean that a claimed composition is not enabled.

> "Enablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. 'The key word is 'undue,' not 'experimentation.'
>
> The determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness, having due regard for the nature of the invention and the state of the art. ... The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *In re Wands*, 8 U.S.P.Q.2d 1400, 1404.

As noted in Wands, the need for routine screening is allowable and does not mean that an invention is not enabled. In the instant case, the fact that antibody positive hybridomas need to be screened for antibodies for the desired reactivity does not mean that the claimed antibodies are not enabled. In the present application, the disclosure teaches that preeclampsia is associated with the magnesium binding defect and that a lower than normal level of peptides correlates with the presence of the defect. Now that such information has been taught by the instant application, the ability to detect a predisposition to preeclampsia has been made a simple matter of using the well known techniques of antibody production.

Furthermore, objective enablement, not actual reduction to practice, is all that is required, as stated by the court in *Fiers v. Revel*, 984 F.2d 1164 (Fed. Cir. 1993):

> [A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. (emphasis in original) 984 F.2d at 1171-1172.

In view of the foregoing, it is urged that the instant specification enables the full breadth of the claims and requests that the rejection under 35 U.S.C. 112, first paragraph, be withdrawn.

In view of the foregoing amendments and remarks, it submitted that the claims remaining for active consideration in this application are in condition for allowance. Accordingly, favorable action at an early date will be appreciated. If the examiner is of the view that any issue remains unresolved, it is respectfully suggested that Applicant's undersigned attorney may be contacted by telephone at the number set forth below.

Respectfully submitted,

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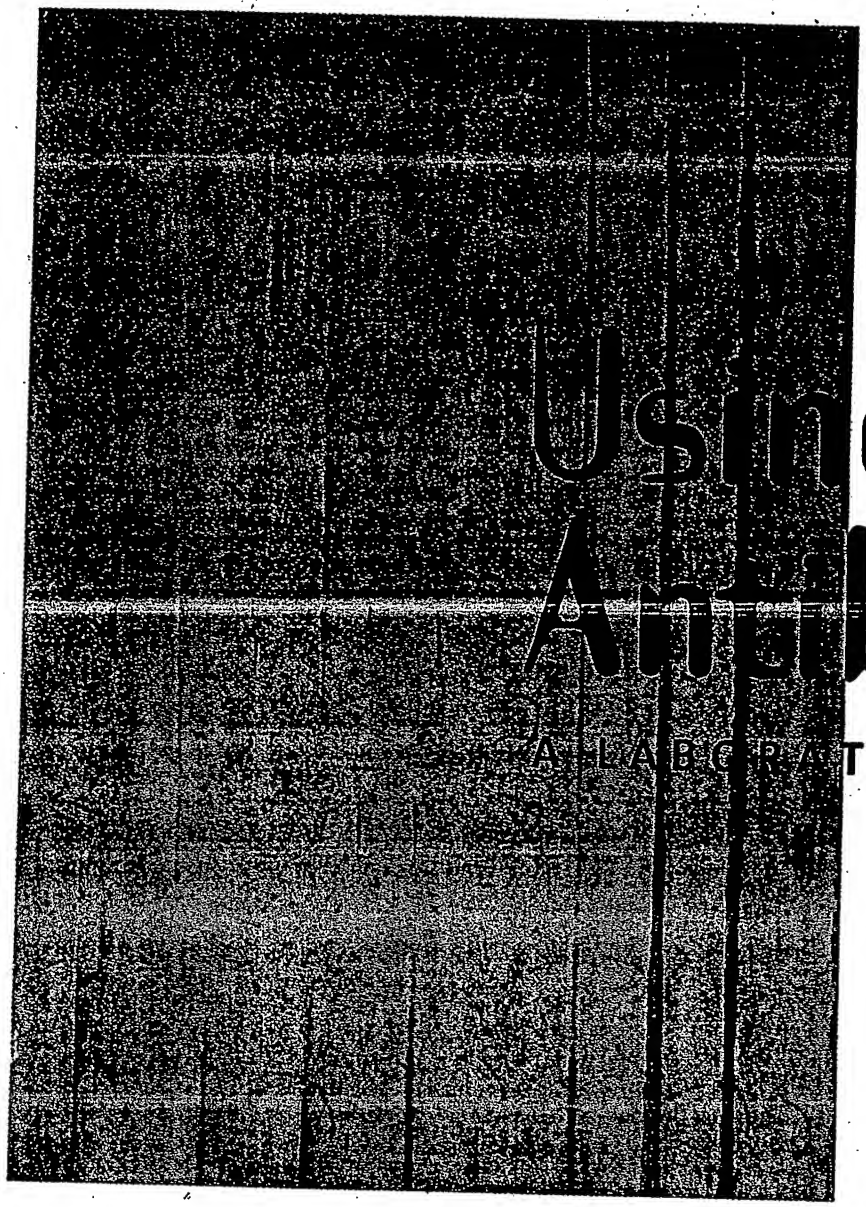

Using Antibodies

A LABORATORY MANUAL

Ed Harlow
MASSACHUSETTS GENERAL HOSPITAL CANCER CENTER
HARVARD MEDICAL SCHOOL

David Lane
DUNDEE UNIVERSITY

COLD SPRING HARBOR LABORATORY PRESS
Cold Spring Harbor, New York

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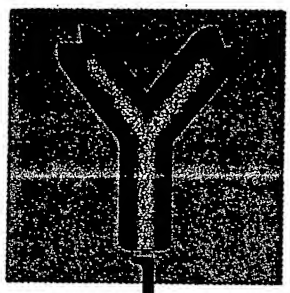
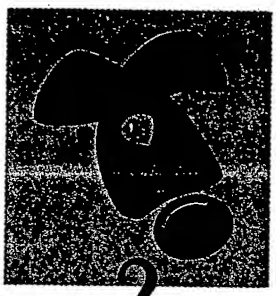


1

Antibody Structure and Function

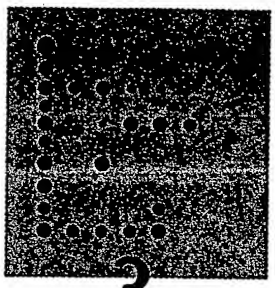
Antibodies are host proteins produced in response to the presence of foreign molecules, organisms, or other agents in the body. Antibodies are synthesized predominantly by plasma cells, a terminally differentiated cell of the B-lymphocyte lineage, and circulate throughout the blood and lymph, where they bind to the antigens. Once formed, the antibody–antigen complexes are removed from circulation mostly through phagocytosis by macrophages. This antibody response is one of the key mechanisms that a host organism uses to protect against the action of foreign molecules or organisms.



2

Antibody–Antigen Interactions

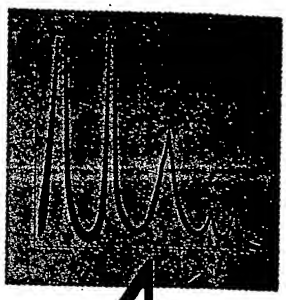
The interaction of an antibody with an antigen forms the basis of all immunochemical techniques. This chapter discusses the properties of the antibody–antigen interaction and is divided into three sections. The first summarizes the structure of the antibody–antigen bonds; the second covers the strength of these interactions, a characteristic known as affinity; and the third presents the factors that contribute to the overall stability of immune complexes, a property called avidity.



3

Choosing Antibodies

Every immunochemical technique presents the antigen in a different physical context. Immunostaining displays the antigen immobilized in its native, but complex, cellular context; immunoprecipitations present the antigen in solution surrounded by huge numbers of other contaminating molecules; immunoblotting leaves the antigen fully denatured and partially purified but bound to a solid support. Not surprisingly, given this diversity of antigen displays, each immuochemical technique requires antibodies with different properties.



4

Handling Antibodies

This chapter presents techniques that are recommended to store, purify, and label antibodies. In general, these techniques are sufficient for handling antibodies that are made in your or your colleagues' labs. Most commercial suppliers who provide antibodies, either labeled or unlabeled, provide suggestions on how to store, handle, and use their antibody products.

Theory

5

Staining Cells

Immunostaining can be used to pinpoint the subcellular localization of a protein antigen, to follow its changing cellular address as cells respond to stimuli, or to compare its locale to other proteins in the same cell. With careful controls, you may also be able to get an idea of how much protein is present in the cell. In this book, the immunostaining procedures have been separated into two major variations based on the source of the cells to be examined. In this chapter, staining cells growing in tissue culture is discussed.

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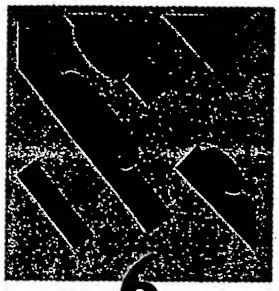

6

Staining Tissues

Immunostaining of tissues of whole organisms can be used to examine the localization of antigens in physiological settings. Using these methods, you can follow an antigen's distribution during development, mark the location of a particular cell type in a multicellular in vivo setting, or determine the presence of an antigen in a diseased tissue. The protocols normally require multiple steps over several days as well as extensive knowledge of the architecture of the tissues being studied. These procedures demand methods to preserve the structure of tissues, which unfortunately are often damaging to the antigens.

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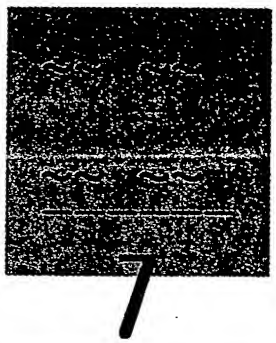

7

Immunoprecipitation

Immunoprecipitation allows the partial purification of antigens, normally proteins, from complex mixtures of soluble molecules. The antigens can be purified up to 10,000-fold by simple and rapid methods that collect the proteins on inert beads. The technique takes about a day and can be combined with any other method that can utilize immobilized proteins as a starting material.

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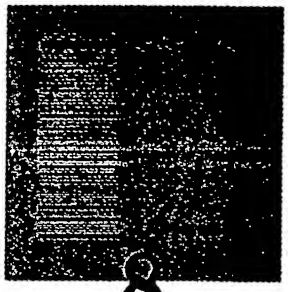

8

Immunoblotting

Immunoblotting provides a reliable method to check any sample for the presence of protein antigen. The assay identifies the protein on the basis of both its interaction with a specific antibody and its relative molecular weight. Immunoblotting can be used to determine other characteristics of your antigen, such as the relative abundance of the antigen or association with other well-characterized antigens. It is also a useful method to characterize new antibody preparations. Immunoblotting involves multiple simple steps that take about a day to complete.

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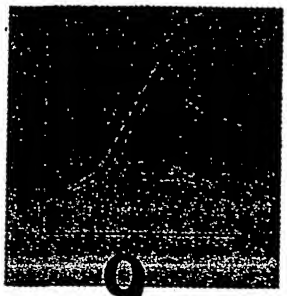
Practice



9

Immunoaffinity Purification

Immunoaffinity purification takes advantage of the high binding affinity and specificity of an antibody for its antigen to allow large quantities of antigen to be isolated in native or near-native states. Not all antibodies are suitable for immunoaffinity purifications, but when a good antibody is available, the procedure is quick and reliable. The procedure can be scaled to any size, takes not more than a half-day to perform, and can achieve levels of purification unmatched by other methods of chromatography.

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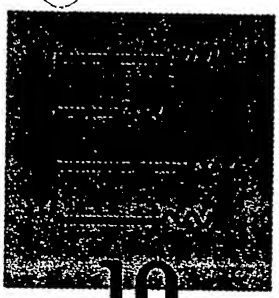

10

Tagging Proteins

Proteins can be tagged using recombinant DNA techniques to allow their ready purification and their specific detection in different host cell systems. These include tags designed to help specifically with detection (such as green fluorescent protein tags) or with purification (such as the His tags and glutathione-S-transferase tags). Epitope tags—short peptide sequences to which strong and specific antibodies have already been produced—can be used for detection and purification using all of the immunological methods described in this book. This chapter describes the different available tag systems, their advantages and disadvantages, and how to choose the right tag for your experiment.

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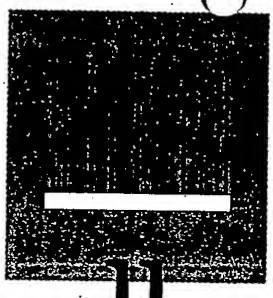

11

Epitope Mapping

Determining the binding sites (epitopes) for monoclonal and polyclonal antigens on protein antigens often provides extremely useful information that greatly extends the power of immunochemical analysis. Epitope mapping is used to examine the specificity of the immune response or to distinguish between different antibodies.

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Appendixes

Appendix I contains information about Electrophoresis; Protein Techniques are covered in Appendix II; and Appendix III provides General Information.

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Practice

Useful Info

Structure of the antibody-antigen complex

The different binding specificities of antibodies for their antigens are established by an elegant series of mechanisms that generate sequence diversity in the combining site of the antibody. These mechanisms include (1) different heavy and light chains combine to form the final antibody; (2) during lymphocyte differentiation genetic recombination brings portions of the CDRs together to form the final transcription unit and produces a large array of different coding regions; (3) imprecise joining during these recombination steps produces new sequence variations; and (4) high rates of somatic mutation allow introduction and selection of new sequences in these regions. The outcome of these variations is a vast array of potential interaction surfaces that can be used to bind to foreign immunogens. The mechanisms that promote this diversity are described in more detail in Chapter 1.

The structure of the antibody–antigen complex has been studied by measuring the affinity of binding between an antibody and a series of related antigens, by using affinity labeling reagents, by site-directed mutagenesis of the antibody combining site, by molecular modeling, and, most compellingly, by X-ray diffraction studies of antibody–antigen cocrystals. Together, these techniques have delineated the region of the antibody molecule that is involved in antigen binding, the region of the antigen molecule that interacts with the antibody, and the molecular basis for antibody specificity.

The antigen-binding site of an antibody is formed by the variable regions of the heavy and light chains

Affinity labeling and X-ray crystallography of immune complexes have established that the antigen-binding site is formed by the heavy- and light-chain variable regions. The two variable regions are tightly associated and are bound to each other by noncovalent interactions (Fig. 2.1). The remainder of the heavy and light chains provide other domains that are not involved in antigen binding but do contribute to how the antibody participates in an immune response (see Chapter 1). The amino acids forming the antigen-binding site are derived from both the heavy and light chains and correspond to the amino acids of the hypervariable regions determined from protein sequencing. The hypervariable regions are known as the complementarity determining regions (CDRs). There are six CDRs, three on the heavy and three on the light chain, and they form discrete loops anchored and oriented by the framework residues of the variable domains (Fig. 2.1).

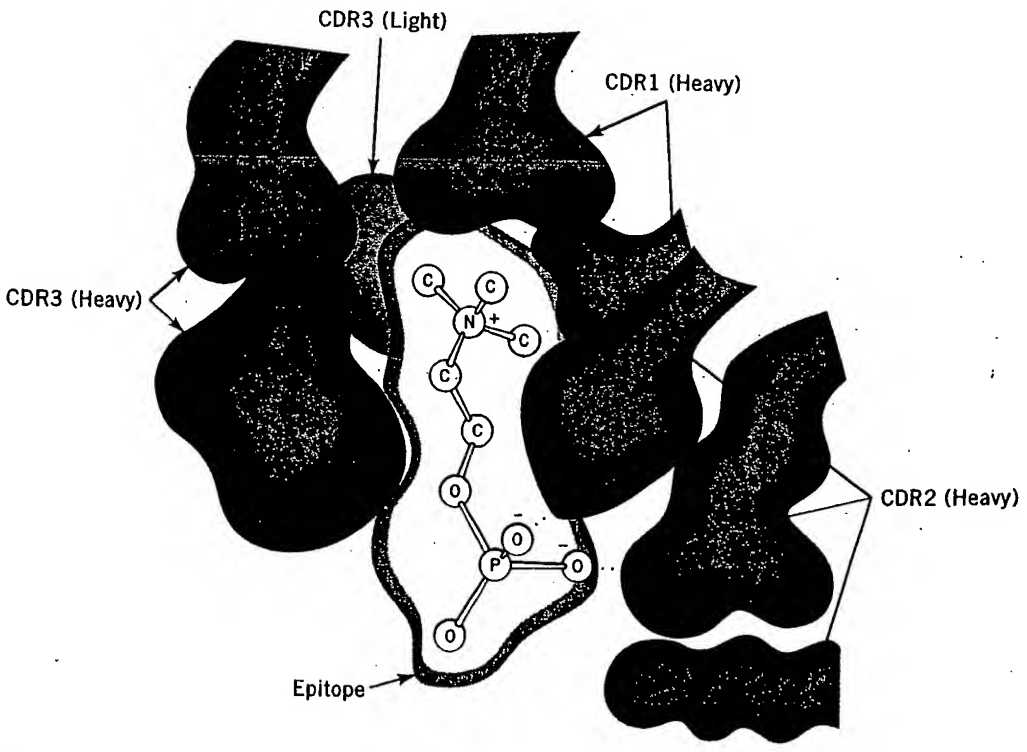

Figure 2.1
The six CDRs form the binding sites for antigen–antibody association. (Adapted from Capra and Edmundson 1977.)

The region of an antigen that binds to an antibody is called an epitope

The region of an antigen that interacts with an antibody is defined as an epitope. An epitope is not an intrinsic property of any particular structure, as it is defined only by reference to the binding site of an antibody. The size of an epitope is governed by the size of the combining site. From X-ray studies of the structures of cocrystals between small antigens bound to antibodies, the size of the combining site was thought to be relatively small. The site was visualized as a cleft or pocket into which the epitope docked. Relatively few of the amino acid side chains of the CDR were in close contact with the antigen. Later work using larger antigens showed that the area of these antigens in close apposition to the antibody can be quite large, occupying as much as 500–750 $Å^2$ and often involving contacts with multiple CDRs, and many times establishing contact with all six. Although these studies have shown that epitopes can be much larger than originally thought, it is still clear that high-affinity antibodies can be raised to small epitopes.

Because antibodies recognize relatively small regions of an entire antigen, occasionally they can find related structures on other molecules. This forms the molecular basis for cross-reaction. Cross-reactions can be helpful in finding related protein family members or distracting when they recognize unrelated proteins with a shared structural feature. For example, cross-reactions can detect highly related structures in common structural regions of protein family members. In this way, an antibody can be a useful tool to identify and study related proteins. However, cross-reactions may also detect similar spatial features in other antigens that do not represent shared structural domains. In these cases, the interactions may still be quite strong but the resulting interactions distracting rather than helpful. Therefore, it is always important to interpret cross-reactions in a conservative manner. Keep in mind that the presence of similar epitopes does not necessarily imply a functional relationship.

Epitopes on protein antigens are local surface structures that can be formed by contiguous or noncontiguous amino acid sequences

Epitopes on an antigen can be formed either by a linear string of amino acid residues or by noncontinguous sequences that are folded into close proximity in the three-dimensional shape on the face of the antigen (Fig. 2.2). A good example of this is seen with one of the lysozyme–antibody cocrystals. Here, the amino acids of lysozyme that form the epitope come from two distant stretches of the primary sequence (residues 18–27 and residues 116–129). Although separated from each other in the primary sequence, these stretches of amino acids are adjacent on the protein surface. At the interface between the antigen and the antibody, a total of 16 amino acids of the antigen make close contacts with 17 amino acids of the antibody, the latter involving all six CDRs. The whole interface is tightly packed and excludes solvent. Strikingly, 748 $Å^2$ or 11% of the surface of lysozyme is covered by the antibody. Similar conclusions come from the study of the second lysozyme–antibody cocrystals and the neuraminidase–antibody cocrystals. Here, either three (lysozyme) or four (neuraminidase) stretches of distant primary sequence form portions of the epitope structure.

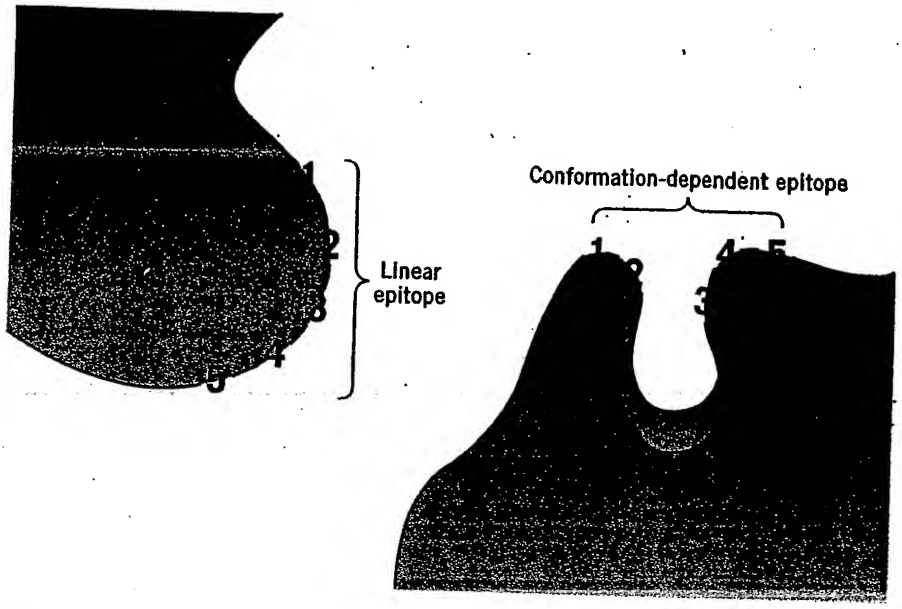

Figure 2.2
Epitopes on an antigen can be formed either by a linear string of amino acid residues or by noncontinguous sequences that are folded into close proximity in the three-dimensional shape on the face of the antigen.

Work with antibodies raised against synthetic peptides or other small antigens provides a set of excellent examples for interactions between antibodies and small, well-defined epitopes. One set of commonly used antibodies that display this property are the anti-phosphotyrosine antibodies, which specifically recognize the phosphorylated side chain of this amino acid in different local regions of many proteins. The ability of antibodies to recognize small epitopes in various structural environments shows the versatility of antibodies to recognize small discrete regions.

Some immune complexes show no alterations in the structure of the antibody or antigen, whereas others show large conformational changes

Antibody–antigen interactions can occur either with large structural changes in the antibody or the antigen or with no detectable changes. From the structures of the first antibody–protein antigen cocrystals, it was clear that both flexible and rigid structures can form good epitopes. In the crystal structure of one of the lysozyme–antibody complexes, no distortion of either the antigen or antibody could be detected, even at high resolution. In sharp contrast, the crystal structure of a neuraminidase–antibody complex revealed substantial structural alterations of both the antigen and antibody. Because the crystallization process itself can induce structural alterations, it is difficult to

prove that these changes are due to antibody binding. However, many other studies have shown that antibodies can induce structural changes in antigens. Good examples of this are the removal of heme from myoglobin and the activation of enzymes by antibody binding.

The antibody–antigen complex is held together by multiple noncovalent bonds

The binding of the antibody to the antigen is entirely dependent on noncovalent interactions, and the antibody–antigen complex is in equilibrium with the free components. The immune complex is stabilized by the combination of weak interactions that depend on the precise alignment of the antigen and antibody. These noncovalent interactions include hydrogen bonds, van der Waals forces, coulombic interactions, and hydrophobic interactions. These interactions can occur between side chains or the polypeptide backbones.

Small changes in antigen structure can affect profoundly the strength of the antibody–antigen interaction. The loss of a single hydrogen bond at the interface can reduce the strength of interaction 1000-fold. The overall interaction is a balance of many attractive and repulsive interactions at the interface. This can be demonstrated in vitro by site-directed mutagenesis. Changing the amino acid residues that form the binding site can alter the strength of an antibody–antigen interaction. This is performed elegantly in vivo by the selection of cells secreting higher-affinity antibodies. By a still poorly understood process, the CDR residues from differentiating clones of B cells undergo extensive mutation, yielding antibodies that differ widely in the microstructure of their antigen-binding sites. Cells that express antibodies with higher affinity are stimulated preferentially to divide. This process continues during the exposure and reexposure to antigen and results in a stronger and more specific antibody response.

Antibodies can bind to a wide range of chemical structures and can discriminate among related compounds

The microenvironment of the combining site can accommodate highly charged as well as hydrophobic molecules. Epitopes composed of carbohydrates, lipids, nucleic acids, amino acids, and a wide range of synthetic organic chemicals have all been identified. The repertoire of possible binding sites is enormous, and antibodies that are specific to novel compounds can be derived readily.

The specificity of antibodies has been demonstrated by a large number of experiments showing that small changes in the epitope structure can prevent antigen recognition. For example, antibodies have been isolated that will differentiate between conformations of protein antigens, detect single amino acid substitutions, or act as weak enzymes by stabilizing transition forms.

tive immunoprecipitations normally require antibody affinities of 10^8 liter mol^{-1} or higher. Affinities of 10^6 to 10^7 liter mol^{-1} may allow the antigen to be detected, but it will not be possible to remove the antigen quantitatively from the solution. These observations mean that some monoclonal antibodies that are positive in other tests may not be usable in immunoprecipitations. The use of polyclonal antibodies or pools of monoclonal antibodies may avoid this problem by interacting with multiple epitopes on the antigen. The effect of antibody affinity on immunoprecipitation is discussed in detail in Chapter 2, and the variables for monoclonal versus polyclonal antibodies are discussed in Chapter 3 and below.

Choosing the correct antibody

Three types of antibody preparations can be used for immunoprecipitations. These are polyclonal antibodies, monoclonal antibodies, and pooled monoclonal antibodies. Their relative advantages and disadvantages are summarized in Table 7.1 and discussed in detail below. Chapter 3 contains a more thorough discussion of the problems of choosing the correct antibody along with a consideration of how to evaluate different antibody sources. The major complicating factors that should influence your choice between available antibodies are the extent of cross-reactions with unrelated antigens, a common problem found with about one-third of monoclonal antibodies, and bad backgrounds, a problem that is more common with polyclonal antibodies. Nonspecific backgrounds can normally be kept to a minimum by titering the amount of antibody to the lowest amount that does not lower the strength of the antigen band (essentially still staying in antibody excess) and by carefully following the preclearing protocols described below.

Table 7.1 *Antibody choice*

	Polyclonal antibodies	Monoclonal antibodies	Pooled monoclonal antibodies
Signal strength	Excellent	Antibody dependent (poor to excellent)	Excellent
Specificity	Usually good, but some background	Excellent, but some cross-reactions	Excellent by avoiding antibodies with cross-reactions
Good features	Stable, multivalent interactions	Specificity Unlimited supply	Stable, multivalent interactions Specificity Unlimited supply
Bad features	Nonrenewable Background	Need high affinity for antigen	Not commonly available

Immunoprecipitations Using Polyclonal Antibodies

Polyclonal antibodies are the most commonly used reagents for immunoprecipitations. Normally they contain antibodies that bind to multiple sites on the antigen and therefore have a much higher avidity for the antigen (see Chapter 2). Having more than one antibody bound to an antigen also has other important advantages. When the immune complexes are collected on any of the solid-phase matrices, such as protein A beads, the availability of multiple binding sites for the protein A molecules provides a more stable antigen–antibody–protein A complex. Together, multiple antibody–antigen interactions and multiple antibody–protein A interactions provide a multivalent complex that is easy to prepare, stable, and can be treated relatively harshly during the washing procedure.

Although using polyclonal antibodies for immunoprecipitations often produces stable multivalent interactions, their use also yields higher nonspecific backgrounds than the use of other types of antibodies. Multiple interactions that lead to forming large complexes are more apt to trap or bind nonspecific proteins. Because polyclonal antibodies normally are used as whole sera, they contain the entire repertoire of circulating antibodies found in the immunized animal at the time the serum was collected. Therefore, serum may contain antibodies that specifically recognize spurious antigens. Because this type of contamination is specific, it cannot be removed by methods that are designed to lower nonspecific background (e.g., preclearing, adding BSA). In these cases, the easiest method to remove these activities is to switch antibody sources. Other antisera are unlikely to contain identical spurious reactions. In some cases, it may also be possible to block the specific antibodies by preincubating the serum with a solution that contains the contaminating proteins (e.g., an acetone powder from a source that does not express the antigen being studied, p. 437).

Because of contaminating activities and increased nonspecific interactions, immunoprecipitations using polyclonal antibodies normally have higher backgrounds than other antibody preparations. Many of these problems are inherent in this technique, but some of the background can be effectively removed by titrating the amount of antisera needed to immunoprecipitate the antigen. By providing the smallest amount of serum necessary for the quantitative recovery of the antigen, the background can be kept to a minimum. In addition, because of the stability of the complexes, nonspecific background problems may be lessened by more stringent washing.

Immunoprecipitations Using Monoclonal Antibodies

The biggest advantage of using monoclonal antibodies for immunoprecipitations is the specificity of their interactions. Because monoclonal antibodies bind to only one epitope, they provide an excellent tool to identify a particular structure on an antigen. Given the right antibody, they can be used not only to detect an antigen, but also to distinguish among different forms of the antigen, including conformational changes or posttranslational modifications. In addition, because the immune complexes formed using monoclonal antibodies are not usually multimeric and are smaller than

those formed when using polyclonal antibodies, there is less of a problem with nonspecific binding. Therefore, the backgrounds are normally cleaner.

Although using monoclonal antibodies for immunoprecipitations may solve or lessen some of the problems found when using polyclonal antibodies, their use also creates another set of difficulties. The most worrisome problem is affinity. Because the antigen is held only by one antibody–antigen interaction (except when the antigen is multimeric), the affinity of the antibody for the antigen is critically important (see discussions of affinity on p. 28). Monoclonal antibodies with affinities lower than about 10^8 liter mol^{-1} are difficult to use in immunoprecipitations. Because many screening techniques for hybridoma fusions detect antibodies with affinities as low as 10^6 liter mol^{-1}, not all monoclonal antibodies work well in immunoprecipitations.

A second problem with using monoclonal antibodies is the possibility of detecting spurious cross-reactions with other polypeptides. Because an epitope can be a relatively small protein structure, often composed of only 4 or 5 amino acids, there is a reasonable chance that a similar epitope can be found on another polypeptide. In some cases, the common epitopes form part of an important structural similarity between antigens, and monoclonal antibodies can be used to detect related antigens. Alternatively, the antibodies may detect small structural similarities confined only to the antibody combining site. This is particularly true for antibodies that recognize denaturation-resistant epitopes. Presumably this occurs because these antibodies recognize features found in the primary structure of the polypeptides. Depending on the set of hybridomas, as many as one in three monoclonal antibodies have been shown to display these types of cross-reactions. Because of the frequency of these cross-reactions, the precipitation of an unexpected polypeptide should be treated as a contaminant until proven otherwise.

Immunoprecipitations using pooled monoclonal antibodies

Using pools of monoclonal antibodies in immunoprecipitation takes advantage of the best properties of both polyclonal and individual monoclonal antibodies. The monoclonal antibodies provide specificity, and the use of multiple antibodies allows the formation of stable multivalent complexes. Consequently, pooled monoclonal antibodies are the best choice of reagents for most immunoprecipitations. Unfortunately, not all antigens have been studied in enough detail to have a set of monoclonal antibodies available for pooling. However, even the use of two antibodies specific for two separate epitopes will greatly increase the avidity for the antigen as well as for protein A or protein G. Therefore, whenever possible, pooled monoclonal antibodies should be used for immunoprecipitations.

Choosing an epitope-mapping method

There are many potential methods for mapping and characterizing the location of epitopes on proteins, ranging from solving the crystal structure of the antibody–antigen complex to analysis of vast libraries of random peptide sequences. These variants are discussed below. Three of the simplest, most widely applicable, and most robust assays are competition assays, gene expression assays, and synthetic peptide-based assays (Table 11.2).

Mapping by competition assay is a very widely used method that can rapidly determine whether two different monoclonal antibodies are able to bind independently to the same protein antigen or whether their binding sites on the protein overlap in such a way that both are not able to bind to the antigen at the same time. The assay can be configured in a large number of different formats using either labeled antigen or labeled antibody. Commonly, the antigen is immobilized on a 96-well plate. The ability of unlabeled antibodies to block the binding of labeled antibodies is measured using radioactive or enzyme labels.

Using this kind of assay with a panel of monoclonal antibodies allows the number of sterically discrete epitopes on the protein antigen to be determined. The method is very versatile and remarkably accurate. For example, we used this kind of assay to map 10 or more independent epitopes on SV40 large T antigen (Gannon and Lane 1990). It is particularly useful in determining if a new monoclonal antibody to a particular protein is distinct from other antibodies to the same protein (Wagener et al. 1983,

Table 11.2 *Application and requirements of recommended epitope-mapping methods*

Method	Conformational epitope	Linear epitope	Requirements	Precision
Competition assay	Yes	Yes	Labeled antibody; antigen	Determines steric competition only
Gene fragment expression	Often but not always	Yes	cDNA must be cloned (gene sequence known)	Conformational 50–200 amino acid domains. Linear 10–20 amino acids
Synthetic peptide library	Never	Yes	Peptide library must be made but cDNA clone not required	Complete description of epitope 3–15 amino acids

1984; Kuroki et al. 1990, 1992a,b). The assay can be used with antibodies that bind both conformational and linear epitopes.

The second approach to mapping an epitope is based on the concept of cutting the protein into smaller fragments and then examining whether the antibody will react with any of these pieces. Historically, these fragments were produced by chemical or proteolytic cleavage of the protein antigen. These remain powerful methods, but the advent of systems for the expression of recombinant proteins has allowed an alternative genetic approach to protein fragmentation. In these procedures, the open reading frame encoding the protein is fragmented either randomly or by specific genetic construction, and the reactivity of the expressed fragments of the protein with the test antibody is determined.

The versatility of DNA fragmentation protocols combined with the vast range of available systems for the expression of recombinant protein has created an almost infinite number of variants on this theme. These range from the entire synthesis of gene segments (Alexander et al. 1992) to the cloning of random fragments of the open reading frame generated by digestion with DNase for expression on the surface of bacteriophage particles (Petersen et al. 1995; Fack et al. 1997).

All of these variants are effective, because only small amounts of protein need to be expressed to determine whether an antibody can bind to it. Thus, even if only a small fraction of the expressed protein fragment folds correctly, it may still be sufficient to give a strong antibody-binding signal, making this method useful for mapping the binding sites of antibodies to both conformation-sensitive and linear epitopes.

A useful example protocol where defined gene fragments are produced by PCR and then transcribed and translated into protein in vitro in the presence of radioactive amino acids is outlined below (p. 391). Binding of the antibody to the labeled protein fragments is then determined by immunoprecipitation and gel electrophoresis.

The third approach to epitope mapping is only applicable to antibodies that work in immunoblotting and react with short linear peptide epitopes. The identification of the epitopes with which these antibodies react has been done using large libraries of random peptide sequence displayed on the surface of phage particles. Alternatively, vast libraries of random synthetic peptides have been analyzed. A much simpler approach in cases where the amino acid sequence of the protein or gene fragment contains the epitope is to synthesize (or order) a defined library of overlapping peptide segments of the protein. These peptide set libraries can then be easily tested for binding to the test antibody in simple binding assays and will define the linear epitope to a stretch of 5–15 amino acids.

5 entries found for *predisposition*.

pre·dis·po·si·tion P **Pronunciation Key** (prē'dĭs-pə-zĭsh'ən)
n.

The state of being predisposed; tendency, inclination, or susceptibility.

pre·dis·po·si·tion (prē'dĭs-pə-zĭsh'ən)
n.

1. The state of being predisposed.
2. A condition of special susceptibility, as to a disease.